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Medullary 5-HT neurons: Switch from tonic respiratory drive to chemoreception during postnatal development

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Abstract

Serotonin (5-HT) neurons contribute to respiratory chemoreception in adult mice, but it is unclear whether they play a similar role in neonatal mice. We studied breathing during development in *Lmx1b^{fl/p}* mice, which lack 5-HT neurons. From postnatal days 1–7 (P1–P7), ventilation of *Lmx1b^{fl/p}* mice breathing room air was 50% of WT mice ($p < 0.001$). By P12, baseline ventilation increased to a level equal to WT mice. In contrast, the hypercapnic ventilatory response (HCVR) of neonatal *Lmx1b^{fl/p}* and WT mice were equal to each other, but were both much less than adult WT mice. By P21 the HCVR of WT mice increased to near adult levels, but the HCVR of *Lmx1b^{fl/p}* mice had not changed, and was 42% less than WT mice. Primary cell cultures were prepared from the ventromedial medulla of neonatal mice, and patch-clamp recordings were made from neurons identified as serotonergic by expression of a reporter gene. In parallel with developmental changes of the HCVR *in vivo*, 5-HT neurons had little chemosensitivity to acidosis until 12 days *in vitro* (DIV), after which their response increased to reach a plateau around 25 DIV. Neonatal *Lmx1b^{fl/p}* mice displayed high mortality and decreased growth rate, and this worsened in hypoxia. Mortality was decreased in hyperoxia. These results indicate that maturation of 5-HT neurons contributes to development of respiratory CO₂/pH chemoreception during the first few weeks of life in mice *in vivo*. A defect in the 5-HT system in early postnatal life decreases survival due in part to hypoxia.

Introduction

5-HT neurons play a role in respiratory control (Al-Zubaidy ZA et al., 1996; Feldman JL et al., 2003; Hodges MR et al., 2008; Niebert M et al., 2011; Pena F & Ramirez JM, 2002; Ptak K et al., 2009) and chemosensory drive (Brust RD et al., 2014; Corcoran AE et al., 2009; Hodges MR & Richerson GB, 2010a; Li A & Nattie E, 2008; Nattie EE et al., 2004; Ray RS

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et al., 2011; Richerson GB, 2004; Teran FA et al., 2014). Their contribution to chemoreception has been characterized in adult mice, but not in neonatal mice. There are a variety of mouse models with dysfunction in their 5-HT system, such as *Pet1*^{-/-} mice that have a 70% reduction of 5-HT neurons, *Lmx1b*^{f/f/p} mice that lack >99% of 5-HT neurons, serotonin transporter (5-HTT) knockout mice that exhibit an absence of 5-HT reuptake and decreased rate of synaptic 5-HT clearance, and others including dietary tryptophan depletion or tryptophan hydroxylase knockout mice (Alenina N et al., 2009; Cummings KJ et al., 2010; Erickson JT et al., 2007; Hodges MR et al., 2009; Holmes A et al., 2003; Li A & Nattie E, 2008; Penatti EM et al., 2011). Some of these mice display respiratory abnormalities, high mortality, and growth retardation in the early postnatal period. *Lmx1b*^{f/f/p} mice allow characterization of the effect of nearly complete absence of 5-HT neurons in the CNS. These mice have severely decreased baseline breathing in the neonatal period. This normalizes during postnatal development (Hodges MR et al., 2009), but these mice display a blunted response to hypercapnia as adults (Hodges MR et al., 2008).

Pet1^{-/-} and *Lmx1b*^{f/f/p} mice both have a low respiratory frequency (f_R) as neonates, but tidal volume (V_T) is lower only in *Lmx1b*^{f/f/p} mice relative to their WT littermates (Hodges MR et al., 2009). There is a reduction in minute ventilation (V_E) in *Pet1*^{-/-} null mice at P4 due to low f_R , but this is compensated by an increase in V_T at P14–15 (Cummings KJ et al., 2010). Similarly, V_E from *Lmx1b*^{f/f/p} mice normalizes around P12, coincident with the age at which apneas cease to occur (Hodges MR et al., 2009). The response to 5% CO₂ has previously been characterized in P4.5 *Pet1*^{-/-} mice, and the change in f_R or V_T were not different compared to age-matched WT mice (Erickson JT et al., 2007). In addition, P5 mice that had previously been deficient in dietary tryptophan during gestation do not display an abnormal response to 7% CO₂ (Penatti EM et al., 2011). It is possible that the chemoreceptive response early in development might not have much dependence on 5-HT neurons, and instead may be elicited primarily by chemosensitive neurons in other central nuclei, such as those in the retrotrapezoid nucleus (RTN) (Kumar NN et al., 2015; Ramanantsoa N et al., 2011; Ruffault PL et al., 2015), locus coeruleus (Johnson SM et al., 2008; Pineda J & Aghajanian GK, 1997) or nucleus tractus solitarius (Dean JB et al., 1990), or by peripheral chemoreceptors. That would be consistent with the findings that the chemosensitivity index (CI) of rat ventromedial medullary neurons increases with age in brain slices and in culture over the first few postnatal weeks (Wang W & Richerson GB, 1999), and that the percentage of medullary 5-HT neurons that are chemosensitive, and the size of their response, is greater in brain slices prepared from mice aged P23–P26 than those aged P14–P18 (Brust RD et al., 2014).

It has previously been shown that neonatal *Lmx1b*^{f/f/p} mice have a decrease in baseline ventilation compared to WT littermates (Hodges MR et al., 2009) and that adult *Lmx1b*^{f/f/p} mice have a decrease in CO₂ chemoreception (Hodges MR et al., 2008), but the effect on chemoreception of genetic deletion of 5-HT neurons has never been examined in neonatal *Lmx1b*^{f/f/p} mice. Here we compared the HCVR of *Lmx1b*^{f/f/p} and WT mice from P1 to P21 to test the hypothesis that there is a switch in the role of 5-HT neurons from providing tonic respiratory drive in neonatal mice, to contributing to the robust HCVR of adult mice. We then used patch clamp recordings from a large number of identified 5-HT neurons *in vitro* to test the hypothesis that there is a developmental increase in cellular chemosensitivity, and

that the age at which this occurs coincides with the age at which there is an increase in the HCVR of WT mice *in vivo*. Finally, we tested the hypothesis that the absence of 5-HT neurons in neonatal *Lmx1b^{fl/p}* mice causes impaired growth rate and decreased survival, and this is due in part to hypoxia caused by the decrease in tonic respiratory drive at this age. Our results confirm that 5-HT neurons are important for baseline breathing in neonatal mice, and show for the first time that they do not play a significant role in central CO₂ chemoreception until they develop intrinsic chemosensitivity after 2–3 postnatal weeks. After that age, the increase in chemosensitivity of 5-HT neurons contributes to the large increase in the HCVR that normally occurs with maturation.

Materials and Methods

All experiments were done in accordance with the guidelines of the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health and were approved by the Yale University and University of Iowa Animal Care and Use Committees.

Animals

Generation of *Lmx1b^{fl/p}* mice, in which nearly all central nervous system 5-HT neurons fail to develop, has previously been described (Zhao Z-Q et al., 2006). These mice are homozygous for floxed *Lmx1b*, and hemizygous for ePet-cre. Generation of ePet-EYFP mice, in which 5-HT neurons express the fluorescent reporter enhanced yellow fluorescent protein (EYFP), has also previously been described (Scott MM et al., 2005).

Genotypes were verified in all mice. Tail samples were obtained at P0–P1, digested and subjected to PCR with the following primers: Flox1: 5′ AGGCTCCATCCATTCTTCTC 3′. Flox2: 5′ CCACAATAAGCAAGAGGCAC 3′. Cre1: 5′ ATTTGCCTGCATTACCGGTGCG 3′. Cre2: 5′ CAGCATTGCTGTCACTTGGTC 3′. YFP1: 5′ GAACTCCAGCAGGACCATGT 3′. YFP2: 5′ TAT ATC ATG GCC GAC AAG CA 3′. PCR products were analyzed via agarose gel electrophoresis. Breeding of *Lmx1b^{fl/p}* and ePet-EYFP mice was as previously reported (Hodges MR et al., 2008; Scott MM et al., 2005).

Whole-body plethysmography

Physiological studies were conducted to measure respiratory frequency (f_R), tidal volume (V_T), ventilation (V_E) and oxygen consumption (VO_2) in awake *Lmx1b^{fl/p}* mice (n=15) and their WT littermates (n=14) from five litters using stop-flow (P1) or flow-through (P4, P7, P12 and P21) whole-animal plethysmography under thermoneutral conditions (30°C) as described in previous studies (Cerpa V et al., 2014; Hodges MR & Richerson GB, 2008; Hodges MR et al., 2009). All experiments were performed in the daytime (light) period. All parameters were determined at each age studied, except VO_2 , which was determined only for P4–P21 as it was not possible to obtain VO_2 for P1 mice due to the small chamber flow rate. The plethysmography chamber gas flow rate was measured using an electronic gas flowmeter (PV500CCMVADA-CP, Cole-Parmer) and PO_2 was measured with an O₂ sensor (S3-A; AEI Technologies). Baseline breathing was measured for more than 30 min in room air (21% O₂ - balance N₂), followed by 10 min in 7% CO₂/21% O₂ (balance N₂). Mice were

returned to their home cage, and approximately 4 hours later were placed in the plethysmograph and exposed for 30 min to room air, followed by 10 min in hypoxia (10% O₂- balance N₂). Data were collected from the last 5 min of each gas exposure. Mice were observed continuously, and data were excluded from analysis during any behaviors that resulted in a breathing pattern other than eupnea (e.g. vocalization, licking, chewing, sniffing, walking, etc.), and were analyzed off-line using software custom-written in Matlab (Matlab, R2007b) from data collected only during episodes of quiet wakefulness (periods with eyes open when mice responded to a mild stimulus such as tapping the chamber). Rectal temperatures were measured in P1–P7 neonates (BAT-12, Physitemp Instruments) before and after experiments. Telemetric temperature probes (IPTT-300, BMDS) were implanted subcutaneously between the scapulae in mice at P8, and then used to measure body temperature during studies of P12 and P21 mice.

Quantification of changes in ventilation

V_T and V_E were calculated using the method of Drorbaugh and Fenn (Drorbaugh JE & Fenn WO, 1955). V_T was calibrated using a small animal ventilator (Minivent, Harvard Apparatus) by injecting 30 – 100 µl of air into the plethysmograph at a rate of 100 strokes/min. Changes in breathing parameters in response to stimuli were quantified in two separate ways: 1) the percentage increase above baseline (e.g. % change in V_E as in Table 1), and; 2) the change in a value induced by a stimulus (e.g. the change in ventilation (V_E) induced by 7% CO₂ as plotted in Fig. 2B).

Cell culture

ePet-EYFP mice (Scott MM et al., 2005) were used to prepare primary dissociated cell cultures so that 5-HT neurons could be visually identified prior to patch clamp recordings. Neonatal (P0 –P2) ePet-EYFP pups were euthanatized by cervical dislocation, and the brainstem was rapidly removed. A wedge of tissue from the ventromedial portion of the rostral half of the medulla (including the raphe pallidus, raphe magnus, and raphe obscurus) was removed as described previously (Wang W et al., 1998). Tissue was digested with papain, triturated, and plated on poly-L-ornithine- and laminincoated coverslips. Cultures were fed and maintained as previously described (Wang W et al., 1998). The age of neurons in culture are expressed as the number of days *in vitro* (DIV).

Patch-clamp recordings

The gramicidin perforated-patch technique was used for electrophysiological recordings. Electrodes (6–14 MΩ; borosilicate glass) were pulled on a micropipette puller (model P-97; Sutter Instrument, Novato, CA) and filled with intracellular solution containing (in mM): 135 KOH, 135 methanesulfonic acid, 10 KCl, 5 HEPES, and 1 EGTA (pH 7.2; 275 ± 5 milliosmolar). Coverslips plated with cultured neurons were transferred to a recording chamber mounted on the stage of an Axiovert 200 inverted microscope (Carl Zeiss USA, Thornwood, NY). The recording chamber was irrigated with aCSF that contained (in mM): 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.3 NaH₂PO₄, 26 NaHCO₃, and 10 dextrose, maintained isocapnic by equilibration with 5% CO₂-95% O₂ (pH 7.4; 305 ± 5 milliosmolar). Hypercapnic acidosis was induced by rapidly changing the bath solution to aCSF of the same composition equilibrated with 9% CO₂-91% O₂ (pH 7.18). Fast glutamatergic,

glycinergic, and GABAergic ionotropic synaptic transmission was blocked by 100 μM picrotoxin (PTX) (Sigma-Aldrich, St. Louis, MO), 50 μM ($\pm$)-2-amino-5-phosphonopentanoic acid (AP-5), and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris, Ellisville, MO). Recordings were performed with a Multiclamp 700B microelectrode amplifier (Molecular Devices, Sunnyvale, CA), and data were collected with a Digidata 1440A data acquisition system and pCLAMP software (Molecular Devices).

Quantification of neuronal response to acidosis

Changes in firing rate of neurons were quantified in two separate ways, as described previously (Wang W et al., 2002): 1) the percentage increase above baseline firing rate, and 2) the Chemosensitivity Index (CI). A neuron was considered to be acidosis-stimulated if it increased its firing rate by more than 20% in response to a decrease in pH of 0.2.

Mouse survival and growth rate

Mortality and growth rate were studied by raising pups from prior to birth (embryonic day 17) to P22 in different environmental conditions, including normoxia (21% O_2), hypoxia (10% O_2), hypercapnic-hypoxia (5% CO_2 /10% O_2), and hyperoxia (50% O_2). Body weight was measured daily from P0–P7 and every other day from P9–P21.

Statistics

Data are presented as mean $\pm$ SEM. The threshold for statistical significance was $p < 0.05$ and determined by one-way or two-way ANOVA followed by *post-hoc* testing with the Dunn or Bonferroni correction for multiple comparisons (Prism 4, GraphPad Software). Survival curves were calculated from P1 to P22 with the Kaplan-Meier method, and groups were compared with the log-rank test. Cox-proportional hazard regression test was used to identify survival-determining factors. The hazard ratio is defined as the ratio of survival of a test group compared to its control group.

Results

5-HT neurons provide essential tonic respiratory drive in early neonatal life

Respiratory parameters were measured during development of WT and *Lmx1b^{f/f/p}* mice, including V_T , f_R , and V_E from P1–P21, and VO_2 from P4–P21. In room air, the breathing pattern of *Lmx1b^{f/f/p}* mice was less stable than WT mice due to variability in frequency and amplitude (Fig. 1A).

In WT mice from P1–P21, V_E in normal room air remained nearly constant when normalized to weight at approximately $2 \text{ ml min}^{-1} \text{ g}^{-1}$ (Fig. 2A). In contrast, P1–P7 *Lmx1b^{f/f/p}* mice showed a lower V_E of $\sim 1 \text{ ml min}^{-1} \text{ g}^{-1}$ (Fig. 2A). The ratio between ventilation and oxygen consumption (V_E/VO_2) was lower in *Lmx1b^{f/f/p}* mice compared to WT littermates at P4 and P7, but equal at P12 and P21 (Fig. 2B). Interestingly, V_E/VO_2 was higher at P12 than other ages for *Lmx1b^{f/f/p}* mice ($p < 0.05$). VO_2 could not be measured at P1 due to the small size of the animals.

The lower V_E in room air in *Lmx1b^{f/f/p}* mice compared to WT mice from P1 to P7 was due to a lower V_T at P1 (Fig. 2C) as well as a lower f_R from P1 to P7 (Fig. 2D). The f_R remained low in *Lmx1b^{f/f/p}* mice at P12 and P21, but was not significantly different than WT mice. In contrast, V_T was higher in *Lmx1b^{f/f/p}* mice at P12 and P21 in comparison to their WT littermates (Fig. 2C). As a result, there was not a significant difference in V_E of *Lmx1b^{f/f/p}* and WT littermates at P12 or P21 (Fig. 2A).

The 5-HT system does not contribute to the response to CO₂ until after P12 in vivo

Upon exposure to 7% CO₂, *Lmx1b^{f/f/p}* and WT mice both increased V_E at each age tested from P1 to P21 (Fig. 3A). In WT mice the V_E response to 7% CO₂ was very small at P1 (Fig. 1B), and there was only a small increase from P4 to P12 (Fig. 3A). It was not until P21 that the increase in V_E of WT mice in response to 7% CO₂ had reached the level typical of adult WT mice ($p < 0.05$; Fig. 3A).

There was no difference in the V_E response to 7% CO₂ of *Lmx1b^{f/f/p}* mice compared to WT mice at any age from P1 to P12, being small in both genotypes (Fig. 1B & 3A). However, the response to 7% CO₂ of *Lmx1b^{f/f/p}* mice did not increase at P21 like it did in WT littermates, so that at that age it was smaller than WT mice by an amount similar to that previously reported for these mice as adults (Hodges MR et al., 2008). Changes in V_E/VO_2 in response to 7% CO₂ followed a similar pattern over this age range (Fig. 3B), being very small at P4 & P7, with no difference between *Lmx1b^{f/f/p}* and WT mice. By P21, the response of WT mice increased to adult levels, whereas that of *Lmx1b^{f/f/p}* mice remained low (Fig. 3B).

When the change of V_E and V_E/VO_2 in response to 7% CO₂ were calculated as the percent change from control values, there was a trend towards a greater response in *Lmx1b^{f/f/p}* mice at younger ages, but it was not significantly different except for V_E at P7 (data not shown). This was due to the lower baseline V_E of *Lmx1b^{f/f/p}* mice compared to WT mice (Fig. 2A). At P21, the change in V_E and V_E/VO_2 of *Lmx1b^{f/f/p}* mice as a percent of control was significantly smaller than that of WT mice. These observations are consistent with calculations of the percent change in V_T and f_R in response to 7% CO₂ at different ages (Table 1).

The 5-HT system does not play a major role in the acute response to sustained hypoxia

In response to 10% O₂, *Lmx1b^{f/f/p}* and WT mice both increased V_E at most ages tested from P1 to P21 (Fig. 1C & 3C). There was a smaller change in V_E in *Lmx1b^{f/f/p}* mice compared to WT mice at P12 and P21 (Fig. 3C). However, there was not a difference in the change in V_E/VO_2 at any age (Fig. 3D). There was also not a significant difference in the percentage change in V_E or V_E/VO_2 in response to 10% O₂ between genotypes at any age (data not shown). These observations are consistent with changes in V_T and f_R as a percent of control in response to 10% O₂ at different ages (Table 1).

Chemosensitivity of 5-HT neurons increases with postnatal age in vitro

In dissociated primary cell cultures prepared from midline medullary tissue obtained from ePet-EYFP mice, chemosensitivity increased with age in culture (Table 2). For example, the

percentage of 5-HT neurons stimulated by acidosis increased with age, being only 11% of 5-HT neurons grown for 1–6 DIV, increasing to 53% of 5-HT neurons 13–21 DIV, and reaching 79% of 5-HT neurons older than 40 DIV (Fig. 4A). The degree of chemosensitivity of 5-HT neurons also increased the longer they were grown in culture, as quantified by either the chemosensitivity index (Fig. 4B) or the percent change in firing rate from baseline induced by acidosis (Fig. 4C). In response to acidosis, the firing rate increased by only $25 \pm 12\%$ at 7.7 DIV, compared to $262 \pm 88\%$ at 46.0 DIV (Fig. 4C). There were not any 5-HT neurons older than 41 DIV that decreased their firing rate by more than 20% in response to acidosis, consistent with previous findings (Wang W et al., 2001), but some young 5-HT neurons were acidosis-inhibited (42% at 1–6 DIV; 5% at 17–21 DIV).

The membrane capacitance (C_m) of 5-HT neurons increased with age (Fig. 5A1), but there was no change in afterhyperpolarization (AHP) amplitude (Fig. 5A2). Resting membrane potential (V_m) did change with age and was approximately -50 mV in 5-HT neurons from mice older than 40 days (Fig. 5B1). There was also an increase in action potential amplitude (measured as the difference between spike threshold and action potential peak) of 5-HT neurons with age (Fig. 5B2).

When compared across all ages, there was no difference in C_m between neurons that were stimulated by acidosis and those that were not (46.77 ± 1.78 and 39.77 ± 2.75 , respectively), and AHP was also the same in acidosis-stimulated and non-chemosensitive neurons (15.4 ± 0.4 and 15.7 ± 0.6 respectively). There were also no differences in resting potential between acidosis-stimulated and non-chemosensitive neurons 47.9 ± 0.4 and 48.7 ± 0.5 , whereas action potential amplitude was larger in neurons that were stimulated by acidosis 83.7 ± 1.4 and 76.6 ± 1.9 respectively.

Growth rate depends on central 5-HT neurons and is decreased by hypoxia

It was previously reported that neonatal *Lmx1b^{f/f/p}* mice have a decrease in growth rate during the first two weeks of life, but then their growth rate recovers at a time that coincides with normalization of baseline ventilation (Hodges MR et al., 2009). It would be expected that a large decrease in baseline ventilation in neonates would also increase mortality, and that there might be a greater effect of hypoxia on mortality and growth rate in neonatal *Lmx1b^{f/f/p}* mice compared to WT mice.

We found that WT and *Lmx1b^{f/f/p}* mice weighed the same at birth, whether they were born in normoxia, hypoxia or hypercapnic hypoxia (Fig. 6). However, their growth rate after birth was influenced by the environmental gas conditions and genotype. The growth rate of *Lmx1b^{f/f/p}* mice was less than that of WT mice in each environmental condition (Fig. 6). Hypoxia (10% O₂) reduced the growth rate of WT mice, making them grow at a rate equal to that of *Lmx1b^{f/f/p}* mice in normoxia, whereas hypoxia further reduced the growth rate of *Lmx1b^{f/f/p}* mice (Fig. 6A). Mice raised in hyperoxia (50% O₂) showed no difference in weight compared to those raised in normoxia for either genotype from birth onward (data not shown). Interestingly, hypercapnic-hypoxia (5% CO₂/10% O₂) increased growth rate in WT mice compared to hypoxia alone (Fig. 6B), but did not alter the effect of hypoxia on growth rate of *Lmx1b^{f/f/p}* mice (data not shown).

Hypoxia increased mortality in *Lmx1b^{ff/p}* mice

Lmx1b^{ff/p} mice had a lower survival rate and higher hazard ratio compared to WT mice in each environmental gas condition (Fig. 7 and Table 3). However, survival curves were significantly different between genotypes only for mice that were raised in hypoxia (Table 3, Cox Proportional Hazard test, effect of genotype condition). Postnatal mortality was increased by hypoxia and decreased by hyperoxia in both WT and *Lmx1b^{ff/p}* mice (Fig. 7A). The lowest survival rate occurred in WT and *Lmx1b^{ff/p}* mice raised in hypoxia (51% and 34%, respectively) and the highest occurred in WT and *Lmx1b^{ff/p}* mice raised in hyperoxia (94% and 86%, respectively). Kaplan-Meier survival curves showed that a high mortality rate occurred in *Lmx1b^{ff/p}* mice for the first 5 postnatal days; only the group raised in hypoxia reflected a significant increase in mortality after that. Mice raised under hypoxic conditions demonstrated a hazard ratio of 3.77 ($p = 0.001$) and 3.36 ($p = 0.0001$) times worse than when raised in normoxia, for WT and *Lmx1b^{ff/p}* mice respectively (Table 3). Hypercapnic-hypoxia led to an improved survival rate compared to hypoxia for WT and *Lmx1b^{ff/p}* mice (Fig. 7B). Hypercapnic-hypoxia resulted in the survival rate being increased to a level that was not statistically different than normoxia for both genotypes (Table 3, Cox Proportional Hazard test, effect of environmental condition).

Discussion

Here we demonstrate a developmental switch in the role of 5-HT neurons in control of breathing. We confirm that during early neonatal life 5-HT neurons provide tonic drive for eupneic respiratory output, as shown previously (Hodges MR et al., 2009; Pena F & Ramirez JM, 2002; Ptak K et al., 2009). We then show that at ages of P12 and younger, 5-HT neurons do not contribute significantly to respiratory CO₂ chemoreception. Subsequently, between P12 and P21 their role in breathing changes so that they no longer provide tonic respiratory drive but now contribute to respiratory chemoreception instead. Development of the HCVR has previously been reported by others in rats (Abu-Shaweesh JM et al., 1999; Bamford OS et al., 1996; Davis SE et al., 2006; Serra A et al., 2001; Stunden CE et al., 2001; Wickstrom R et al., 2002) and mice (Ramanantsoa N et al., 2011). Here we found that there was a large increase in respiratory chemoreception in WT mice *in vivo* over the first few postnatal weeks. The HCVR of WT mice was small at P1, P4 & P7, showed a small increase at P12 compared to P7, and then became even larger by P21. Maturation of respiratory chemoreception *in vivo* was paralleled by maturation of chemosensitivity of 5-HT neurons *in vitro*. There was minimal chemosensitivity of 5-HT neurons in cell culture up to the age of 12 DIV, followed by rapid development of a response to acidosis between 12 DIV and 21 DIV, and after 25 DIV cellular chemosensitivity reached a plateau. These results suggest that maturation of chemosensitivity of 5-HT neurons is an important contributor to maturation of respiratory chemoreception *in vivo*. Consistent with a role of 5-HT neurons in contributing to respiratory chemoreception, genetic deletion of 5-HT neurons had no effect on the HCVR at P12 or earlier, an age at which 5-HT neurons have minimal response to acidosis *in vitro*, but did lead to a decrease in the HCVR at P21, an age at which 5-HT neurons express a robust response to acidosis *in vitro*.

5-HT neurons and tonic respiratory drive in neonatal mice

The baseline ventilatory parameters observed here in neonatal WT mice were similar to those reported previously (Erickson JT et al., 2007; Eugenin J et al., 2008; Robinson DM et al., 2002). There were changes in V_T and f_R across development, but V_E remained relatively constant at $2 \text{ ml min}^{-1} \text{ g}^{-1}$. As shown previously (Erickson JT et al., 2007; Hodges MR et al., 2009), genetic deletion of 5-HT neurons led to disruption of rhythmic ventilation at birth, with V_E of P1–P7 *Lmx1b^{f/f/p}* mice in normoxia less than 50% of that in WT mice. However, as *Lmx1b^{f/f/p}* mice matured, baseline breathing normalized, so that there was no difference in baseline V_E compared to WT mice by the time they were P12. There was an increase in V_T in *Lmx1b^{f/f/p}* mice compared to WT mice at P12 & P21, which could be due to an undefined compensatory effect of loss of 5-HT neurons during early embryonic life, as previously proposed (Erikson et al., 2007), and would correlate with the age that the serotonergic system switches from tonic drive to chemoreception as supported by the current work. It has previously been shown that 5-HT neuron output is required to maintain normal hypoglossal nerve root bursting in neonatal brainstem slices (Erickson JT et al., 2007; Hodges MR et al., 2009; Pena F & Ramirez JM, 2002; Ptak K et al., 2009; Ramirez JM & Viemari JC, 2005) and in a neonatal en bloc brainstem preparation *in vitro* (Hodges MR et al., 2009). These data indicate that 5-HT neurons provide tonic drive to the respiratory network prior to P12, but after mice mature 5-HT neurons are not required for normal V_E , possibly because other modulatory inputs develop by that age or compensate for the loss of 5-HT neurons. 5-HT neurons project to the major respiratory nuclei (Ellenberger HH, 1999; Holtman JR, 1988; Holtman JR et al., 1990; Ptak K et al., 2009), and electrical, chemical and optogenetic stimulation of 5-HT projections increases phrenic nerve activity in adult animals (DePuy SD et al., 2011; Holtman JR et al., 1986; Lalley PM et al., 1997; Richerson GB, 2004).

Our data are consistent with the conclusion that 5-HT neurons provide essential tonic drive for breathing in awake behaving animals during the early postnatal period. In adults, 5-HT neurons may not be required for maintaining normal V_E at baseline, but can still stimulate breathing above baseline levels.

Development of the HCVR in rodents

There have been several previous studies that systematically compared the ventilatory response to CO_2 of rats *in vivo* at different ages during development (Abu-Shaweesh JM et al., 1999; Bamford OS et al., 1996; Bravo K et al., 2016; Davis SE et al., 2006; Serra A et al., 2001; Stunden CE et al., 2001; Wickstrom R et al., 2002). These studies have consistently found that there is maturation of the HCVR during the first few weeks of life. In studies that included animals over a large enough age range, it was also consistently found that there is a nadir of the response between P5–P9 and that the amplitude of the HCVR did not increase to the levels seen in adults until P18–P23 (Bamford OS et al., 1996; Davis SE et al., 2006; Serra A et al., 2001; Stunden CE et al., 2001).

There have been differences in results obtained during the first few days of life in rats. One study reported that the HCVR is larger in P1–P3.5 rats than in adults (>P100) (Stunden CE et al., 2001), whereas two other studies reported that there was only a 20–25% increase in

V_E in response to inhalation of 7% CO_2 in P1 rats compared to a 100–150% increase in V_E in adults (Davis SE et al., 2006; Serra A et al., 2001). In two additional studies in rats, it was also found that the HCVR at P1–P3 was very small (Bamford OS et al., 1996; Wickstrom R et al., 2002).

There have been fewer studies of the HCVR in mice over the full range of postnatal development. One study examined mice from P2–P21.5, and as with rats the HCVR was smaller during the first week than at older ages, although the response increased at P12.5 to a level as large as that at P21.5 (Bissonnette JM & Knopp SJ, 2004). In a different study, V_E increased in P2 WT mice by approximately 80% in response to 8% CO_2 , whereas at P9 the response was slightly less (about 70%), before increasing to near 150% at P22 and near 250% at 4 months (Ramanantsoa N et al., 2011). A study of WT mice at P0 documented a 61% increase in V_E in response to 8% CO_2 (Ruffault PL et al., 2015), whereas one in P2 WT mice documented a response to 8% CO_2 that was dependent on ambient temperature, and was 46–61% at 29 C and 96–116% at 35 C (Ramanantsoa N et al., 2007).

Taken together, the above results are in general agreement as showing a relatively small HCVR in rats and mice from P5–P12, an increase in the HCVR from P12–P22, and a plateau shortly after P28 (Putnam RW et al., 2005). There is more variability in the reported HCVR from P0–P3, although that is an age at which it is technically difficult to obtain accurate measurements of tidal volume. Our results in WT mice *in vivo* are generally consistent with these previously published data.

Role of 5-HT neurons in the HCVR

A subpopulation of 5-HT neurons contributes to the HCVR in adult animals *in vivo* (Belegu R et al., 1999; Bernard DG et al., 1996; Corcoran AE et al., 2009; Hodges MR et al., 2008; Johnson PL et al., 2005; Nattie EE et al., 2004; Ray RS et al., 2011; Teppema LJ et al., 1997; Teran FA et al., 2014; Veasey SC et al., 1995; Wang W et al., 1998; Wang W & Richerson GB, 1999; Wang W et al., 2001). The HCVR is decreased after genetic deletion of 5-HT neurons in adult *Lmx1b^{fl/p}* mice (Hodges MR et al., 2008). It is also decreased in adult mice after activation of inhibitory DREADD receptors expressed either on the majority of 5-HT neurons (Ray RS et al., 2011) or only on the subset of 5-HT neurons derived from rhombomeres 3 & 5 (Brust RD et al., 2014). These and other data support a role of 5-HT neurons in contributing to the HCVR in adults (Teran FA et al., 2014). However, there is no evidence that 5-HT neurons play an important role in the HCVR of neonatal rodents. The current findings indicate that 5-HT neurons do not play a role in the HCVR until after P12, as the response to hypercapnia in *Lmx1b^{fl/p}* mice was not significantly different from that in WT mice at P12 or younger. In contrast, the HCVR was 50% of that in WT mice at P21, which is similar to what has been reported for adult mice (Hodges MR et al., 2008). These results are in general agreement with data showing that fluoxetine exposure *in utero* blunts the HCVR of mature mice much more than that of neonatal mice (Bravo K et al., 2016).

Role of 5-HT neuron chemosensitivity

The cellular mechanisms of central respiratory chemoreception are controversial, with a number of cell types proposed as CO_2 /pH sensors, including rhombomere 5 derived 5-HT

neurons in the medulla (Brust RD et al., 2014), Phox2b expressing neurons in the RTN (Kumar NN et al., 2015), astrocytes in the ventrolateral medulla (Spyer KM & Thomas T, 2000) and neurons in a number of other regions including the nucleus tractus solitarius (Dean JB et al., 1990) and locus coeruleus (Gargaglioni LH et al., 2010; Johnson SM et al., 2008; Pineda J & Aghajanian GK, 1997). However, there is no evidence that the mechanisms involved are uniform at all ages or conditions.

5-HT neurons have been proposed to contribute to detection of CO₂ in adult rodents (Brust RD et al., 2014; Corcoran AE et al., 2009; Hodges MR & Richerson GB, 2010b; Li A & Nattie E, 2008; Nattie EE et al., 2004; Ray RS et al., 2011; Richerson GB, 2004; Teran FA et al., 2014). There are data obtained from neonatal mice that have led some investigators to the conclusion that 5-HT neurons do not play a role in respiratory chemoreception (Erickson JT et al., 2007; Mulkey DK et al., 2007a; Mulkey DK et al., 2007b; Penatti EM et al., 2011; Ramanantsoa N et al., 2011). However, the current data indicate that experiments from neonatal mice do not allow study of the mechanisms involved in chemoreception in adults. Instead, it appears that there is developmental maturation of the HCVR in rodents from a small response in the first week of life to a much more sensitive response after 2–3 weeks. This switch to a larger chemosensitive ventilatory response coincides with the age of appearance of a large response of 5-HT neurons to acidosis in brain slices and cell culture (Brust RD et al., 2014; Wang W & Richerson GB, 1999).

The possibility of a developmental switch in the mechanisms of chemoreception is consistent with data from mice in which there is targeted deletion of RTN neurons (Ramanantsoa N et al., 2011). At the ages of P2 and P9 there is essentially complete absence of the HCVR in the absence of an RTN. However, at P22 an HCVR appears, and by adulthood the response is approximately 50% of that in WT mice despite the absence of an RTN. The age at which the HCVR emerges in these mice coincides with the age at which chemosensitivity develops in 5-HT neurons. Taken together, the results suggest that in adult mice the RTN and 5-HT neurons both contribute to the ventilatory response to CO₂, since deletion of either group of neurons leads to a 50% decrease in the HCVR (Hodges MR et al., 2008; Ramanantsoa N et al., 2011). Previous studies assessing the role of 5-HT neurons in the response to CO₂ in young mice with a deficient 5-HT system (Erickson JT et al., 2007; Mulkey DK et al., 2007a; Mulkey DK et al., 2007b; Penatti EM et al., 2011; Ramanantsoa N et al., 2011) did not consider the possibility that 5-HT neurons had not yet fully developed chemosensitivity.

The existing data indicate that chemosensitivity is not mediated by 5-HT neurons alone. For example, the HCVR is still 50% of normal despite the near absence of 5-HT neurons in *Lmx1b^{fl/p}* mice (Hodges MR et al., 2008). The HCVR in these mice can be restored to normal by intracerebroventricular infusion of 5-HT (Hodges MR et al., 2008), indicating that 5-HT can enhance chemosensitivity of nonserotonergic chemoreceptors, such as those in the RTN, NTS or locus coeruleus, or can enhance synaptic transmission in central afferent pathways mediating inputs from peripheral chemoreceptors. This effect of 5-HT in enhancing other chemoreceptors is not incompatible with a co-existing role of chemosensitivity by 5-HT neurons, a hypothesis that is supported by a large body of evidence (Richerson GB, 2004; Teran FA et al., 2014).

In vitro study of 5-HT neuron chemosensitivity

Publications using patch clamp recordings to study rodent 5-HT neurons in brain slices have used animals that were younger than P12 (Abbott SB et al., 2011; Bayliss DA et al., 1997; Bayliss DA et al., 1995; Mulkey DK et al., 2007a; Mulkey DK et al., 2004; Mulkey DK et al., 2007b), but as shown here and previously (Brust RD et al., 2014; Wang W & Richerson GB, 1999) 5-HT neurons have a very small response to acidosis before P12. It is difficult to prepare viable slices of the medulla after P12 compared to other brain regions such as the hippocampus (Richerson GB & Messer C, 1995), possibly due to the admixture of fiber tracts and small nuclei. In addition, expression of myelin increases from P14 to P21, which degrades visibility of neurons using differential interference contrast (DIC) microscopy, making it difficult to visualize 5-HT neurons to obtain stable patch clamp recordings for the long periods of time needed to accurately document chemosensitivity (typically more than 1 hour). For these reasons, it has not been possible to use brain slices to quantify chemosensitivity of large numbers of 5-HT neurons across the full developmental range from P1 to adulthood.

Here we quantified chemosensitivity of 5-HT neurons that were isolated from P0–P2 mice, and then grown in dissociated cell culture. There is a theoretical concern that neurons in culture might revert to an immature state (Landis S, 1985), but that does not appear to be the case here. Others have concluded that “neurons in dissociated cultures appear to retain their individual identities, presumably because they are postmitotic and committed in their differentiation at the time they are introduced in culture” (Banker G & Goslin K, 1998). We have previously shown that 5-HT neurons in culture retain their expression of tryptophan hydroxylase, and their basic electrophysiological signature (Wang W et al., 2001). Here we found that 5-HT neurons in culture developed chemosensitivity with a very similar age dependence as 5-HT neurons in brain slices over the first 2–3 postnatal weeks, with acidosis causing only small changes in firing rate of neonatal 5-HT neurons, and then chemosensitive responses progressively increase over the first few weeks of age (Brust RD et al., 2014; Wang W & Richerson GB, 1999). Neurons in culture were healthier and patch clamp recordings were more stable compared to brain slices, allowing quantification of chemosensitivity to be extended *in vitro* to ages corresponding to full maturity *in vivo*. In our previous work on development of chemosensitivity (Wang W & Richerson GB, 1999), we only recorded from a small number of neurons (24 acidosis stimulated) whose neurotransmitter was not identified, and it is estimated that only 10–20% of those neurons were serotonergic. Therefore, that approach did not allow determination of the percentage of 5-HT neurons that were chemosensitive at any given age. In contrast, recordings made here were exclusively from 5-HT neurons, allowing quantification of the percentage of 5-HT neurons that were chemosensitive at ages from P1 to P70, as well as quantification of the response of a purified population of 5-HT neurons to acidosis. Our results indicate that chemosensitivity increases in culture with a similar time course as in brain slices, with a plateau that is reached at some point on or after P25.

Correlation between chemosensitivity of 5-HT neurons and the HCVR

The use of a reporter (YFP) to identify 5-HT neurons allowed us to identify a close correlation between the age dependence of chemosensitivity of 5-HT neurons *in vitro* and

maturation of the HCVR in mice *in vivo*. Both are small during the first two weeks and reach a mature level after 3–4 weeks. This suggests a possible mechanistic link between the two phenomena. Further evidence supporting cause and effect was the finding that genetic deletion of 5-HT neurons had little effect on the HCVR *in vivo* during the first two weeks, and resulted in a decrease in the HCVR only after 3 weeks. Taken together, these data are consistent with the hypothesis that the developmental increase in the HCVR *in vivo* is due at least in part to a developmental increase in chemosensitivity of 5-HT neurons.

5-HT, growth retardation and mortality

Lmx1b^{ff/p} mice showed impaired growth compared to WT mice in each gas condition tested. Interestingly, growth retardation was worsened by hypoxia, and WT mice raised in hypoxia grew at a rate parallel to *Lmx1b^{ff/p}* mice raised in normoxia, suggesting that a defect in the 5-HT system has detrimental effects on growth comparable to being raised in a hypoxic environment. This is consistent with the possibility that the decreased ventilation in neonatal *Lmx1b^{ff/p}* mice leads to growth retardation due to hypoxia. Hypercapnic hypoxia improved growth rate compared to normocapnic hypoxia in WT mice, but did not affect growth in *Lmx1b^{ff/p}* mice.

Mortality was also increased by hypoxia in both WT and *Lmx1b^{ff/p}* mice, and *Lmx1b^{ff/p}* mice were relatively more sensitive to low O₂, again consistent with *Lmx1b^{ff/p}* mice suffering from hypoxia at baseline. Hypercapnic hypoxia improved survival compared to normocapnic hypoxia in both WT and *Lmx1b^{ff/p}* mice.

These results show that a defect in the 5-HT system causes decreased ventilation and increases vulnerability to hypoxia in mice during early postnatal life. Defects in the 5-HT system have previously been shown to increase mortality to hypoxia in neonatal rodents (Cummings KJ et al., 2011). Our results could be related to the ventilatory abnormalities and susceptibility to exogenous stressors proposed to occur in sudden infant death syndrome (SIDS). SIDS has been associated with brainstem 5-HT abnormalities (Broadbelt KG et al., 2012; Duncan JR et al., 2010; Kinney HC et al., 2005; Kinney HC et al., 2003; Kinney HC et al., 2009; Kinney HC & Thach BT, 2009; Ozawa Y & Okado N, 2002; Paterson DS et al., 2010; Paterson DS et al., 2006). The current results demonstrate that neonatal mice lacking 5-HT neurons are more susceptible to an exogenous stressor, and have an increased risk of postnatal death. The timing of the developmental switch from tonic drive to respiratory chemoreception in the postneonatal period may define a period of instability of breathing. It is tempting to think this might coincide with a time of increased risk for SIDS in human infants, which peaks at 4–6 months rather than in newborns (Kinney HC et al., 2009).

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References

Abbott SB, Kanbar R, Coates MB, Stornetta RJ, Guyenet PG. C1 neurons excite locus coeruleus and A5 noradrenergic neurons along with the sympathetic outflow. *FASEB Journal*. 2011;25.

- Abu-Shaweesh JM, Dreshaj IA, Thomas AJ, Haxhiu MA, Strohl KP, Martin RJ. Changes in respiratory timing induced by hypercapnia in maturing rats. *J Appl Physiol* (1985). 1999; 87(2):484–490. [PubMed: 10444602]
- Al-Zubaidy ZA, Erickson RL, Greer JJ. Serotonergic and noradrenergic effects on respiratory neural discharge in the medullary slice preparation of neonatal rats. *Pflugers Arch Eur J Physiol*. 1996; 431(6):942–949. [PubMed: 8927513]
- Alenina N, Kikic D, Todiras M, Mosienko V, Qadri F, Plehm R, Boye P, Vilianovitch L, Sohr R, Tenner K, Hoertnagl H, Bader M. Growth retardation and altered autonomic control in mice lacking brain serotonin. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106(25):10332–10337. [PubMed: 19520831]
- Bamford OS, Schuen JN, Carroll JL. Effect of nicotine exposure on postnatal ventilatory responses to hypoxia and hypercapnia. *Respir Physiol*. 1996; 106(1):1–11. [PubMed: 8946572]
- Banker, G., Goslin, K. Types of Nerve Cell Cultures, Their Advantages and Limitations. In: Banker, G., Goslin, K., editors. *Culturing Nerve Cells*. 2. Cambridge, MA: MIT Press; 1998. p. 11-35.
- Bayliss DA, Li YW, Talley EM. Effects of serotonin on caudal raphe neurons: Activation of an inwardly rectifying potassium conductance. *J Neurophysiol*. 1997; 77(3):1349–1361. [PubMed: 9084602]
- Bayliss DA, Umekiya M, Berger AJ. Inhibition of N-Type and P-Type calcium currents and the after hyperpolarization in rat motoneurons by serotonin. *J Physiol-London*. 1995; 485(3):635–647. [PubMed: 7562606]
- Belegu R, Hadziefendic S, Dreshaj IA, Haxhiu MA, Martin RJ. CO₂-induced c-fos expression in medullary neurons during early development. *Resp Physiol*. 1999; 117(1):13–28.
- Bernard DG, Li AH, Nattie EE. Evidence for central chemoreception in the midline raphe. *J Appl Physiol*. 1996; 80(1):108–115. [PubMed: 8847290]
- Bissonnette JM, Knopp SJ. Hypercapnic ventilatory response in mice lacking the 65 kDa isoform of Glutamic Acid Decarboxylase (GAD65). *Respir Res*. 2004; 5:3. [PubMed: 15134589]
- Bravo K, Eugenin JL, Llona I. Perinatal fluoxetine exposure impairs the CO₂ chemoreflex: implications for sudden infant death syndrome. *Am J Respir Cell Mol Biol*. 2016
- Broadbelt KG, Rivera KD, Paterson DS, Duncan JR, Trachtenberg FL, Paulo JA, Stapels MD, Borenstein NS, Belliveau RA, Haas EA, Stanley C, Krous HF, Steen H, Kinney HC. Brainstem deficiency of the 14-3-3 regulator of serotonin synthesis: A proteomics analysis in the sudden infant death syndrome. *Mol Cell Proteomics*. 2012; 11(1)
- Brust RD, Corcoran AE, Richerson GB, Nattie E, Dymecki SM. Functional and developmental identification of a molecular subtype of brain serotonergic neuron specialized to regulate breathing dynamics. *Cell Rep*. 2014; 9(6):2152–2165. [PubMed: 25497093]
- Cerpa V, Gonzalez A, Richerson GB. Diphtheria toxin treatment of Pet-1-Cre floxed diphtheria toxin receptor mice disrupts thermoregulation without affecting respiratory chemoreception. *Neuroscience*. 2014; 279:65–76. [PubMed: 25171790]
- Corcoran AE, Hodges MR, Wu Y, Wang W, Wylie CJ, Deneris ES, Richerson GB. Medullary serotonin neurons and central CO₂ chemoreception. *Respir Physiol Neurobiol*. 2009; 168(1–2):49–58. [PubMed: 19394450]
- Cummings KJ, Hewitt JC, Li A, Daubenspeck JA, Nattie EE. Postnatal loss of brainstem serotonin neurones compromises the ability of neonatal rats to survive episodic severe hypoxia. *J Physiol*. 2011; 589(Pt 21):5247–5256. [PubMed: 21911619]
- Cummings KJ, Li A, Deneris ES, Nattie EE. Bradycardia in serotonin-deficient Pet-1(–/–) mice: influence of respiratory dysfunction and hyperthermia over the first 2 postnatal weeks. *Am J Physiol Regul Integr Comp Physiol*. 2010; 298(5):R1333–R1342. [PubMed: 20421636]
- Davis SE, Solhied G, Castillo M, Dwinell M, Brozoski D, Forster HV. Postnatal developmental changes in CO₂ sensitivity in rats. *J Appl Physiol*. 2006
- Dean JB, Bayliss DA, Erickson JT, Lawing WL, Millhorn DE. Depolarization and stimulation of neurons in nucleus tractus solitarius by carbon dioxide does not require chemical synaptic input. *Neuroscience*. 1990; 36(1):207–216. [PubMed: 2120613]
- DePuy SD, Kanbar R, Coates MB, Stornetta RL, Guyenet PG. Control of breathing by raphe obscurus serotonergic neurons in mice. *J Neurosci*. 2011; 31(6):1981–1990. [PubMed: 21307236]

- Drorbaugh JE, Fenn WO. A barometric method for measuring ventilation in newborn infants. *Pediatrics*. 1955; 16(1):81–87. [PubMed: 14394741]
- Duncan JR, Paterson DS, Hoffman JM, Mokler DJ, Borenstein NS, Belliveau RA, Krous HF, Haas EA, Stanley C, Nattie EE, Trachtenberg FL, Kinney HC. Brainstem serotonergic deficiency in sudden infant death syndrome. *JAMA*. 2010; 303(5):430–437. [PubMed: 20124538]
- Ellenberger HH. Nucleus ambiguus and bulbospinal ventral respiratory group neurons in the neonatal rat. *Brain Res Bull*. 1999; 50(1):1–13. [PubMed: 10507466]
- Erickson JT, Shafer G, Rossetti MD, Wilson CG, Deneris ES. Arrest of 5HT neuron differentiation delays respiratory maturation and impairs neonatal homeostatic responses to environmental challenges. *Respir Physiol Neurobiol*. 2007; 159(1):85–101. [PubMed: 17656160]
- Eugenin J, Otarola M, Bravo E, Coddou C, Cerpa V, Reyes-Parada M, Llona I, von Bernhardt R. Prenatal to early postnatal nicotine exposure impairs central chemoreception and modifies breathing pattern in mouse neonates: A probable link to sudden infant death syndrome. *J Neurosci*. 2008; 28(51):13907–13917. [PubMed: 19091979]
- Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci*. 2003; 26:239–266. [PubMed: 12598679]
- Gargaglioni LH, Hartzler LK, Putnam RW. The locus coeruleus and central chemosensitivity. *Respir Physiol Neurobiol*. 2010; 173(3):264–273. [PubMed: 20435170]
- Hodges MR, Richerson GB. Interaction between defects in ventilatory and thermoregulatory control in mice lacking 5-HT neurons. *Respir Physiol Neurobiol*. 2008; 164(3):350–357. [PubMed: 18775520]
- Hodges MR, Richerson GB. Medullary serotonin neurons and their roles in central respiratory chemoreception. *Respir Physiol Neurobiol*. 2010a; 173(3):256–263. [PubMed: 20226279]
- Hodges MR, Richerson GB. The role of medullary serotonin (5-HT) neurons in respiratory control: contributions to eupneic ventilation, CO₂ chemoreception, and thermoregulation. *J Appl Physiol*. 2010b; 108(5):1425–1432. [PubMed: 20133432]
- Hodges MR, Tattersall GJ, Harris MB, McEvoy SD, Richerson DN, Deneris ES, Johnson RL, Chen ZF, Richerson GB. Defects in breathing and thermoregulation in mice with near-complete absence of central serotonin neurons. *J Neurosci*. 2008; 28(10):2495–2505. [PubMed: 18322094]
- Hodges MR, Wehner M, Aungst J, Smith JC, Richerson GB. Transgenic mice lacking serotonin neurons have severe apnea and high mortality during development. *J Neurosci*. 2009; 29(33):10341–10349. [PubMed: 19692608]
- Holmes A, Murphy DL, Crawley JN. Abnormal behavioral phenotypes of serotonin transporter knockout mice: Parallels with human anxiety and depression. *Biol Psychiatry*. 2003; 54(10):953–959. [PubMed: 14625137]
- Holtman JR. Immunohistochemical localization of serotonin-containing and substance P-containing fibers around respiratory muscle motoneurons in the nucleus ambiguus of the cat. *Neuroscience*. 1988; 26(1):169–178. [PubMed: 2458541]
- Holtman JR, Anastasi NC, Norman WP, Dretchen KL. Effect of electrical and chemical-stimulation of the raphe obscurus on phrenic-nerve activity in the cat. *Brain Res*. 1986; 362(2):214–220. [PubMed: 2867815]
- Holtman JR, Marion LJ, Speck DF. Origin of serotonin-containing projections to the ventral respiratory group in the rat. *Neuroscience*. 1990; 37(2):541–552. [PubMed: 2133358]
- Johnson PL, Hollis JH, Moratalla R, Lightman SL, Lowry CA. Acute hypercarbic gas exposure reveals functionally distinct subpopulations of serotonergic neurons in rats. *J Psychopharmacology*. 2005; 19(4):327–341.
- Johnson SM, Haxhiu MA, Richerson GB. GFP-expressing locus ceruleus neurons from Prp57 transgenic mice exhibit CO₂/H⁺ responses in primary cell culture. *J Appl Physiol*. 2008; 105(4):1301–1311. [PubMed: 18635881]
- Kinney HC, Myers MM, Belliveau RA, Randall LL, Trachtenberg FL, Ten Fingers S, Youngman M, Habbe D, Fifer WP. Subtle autonomic and respiratory dysfunction in sudden infant death syndrome associated with serotonergic brainstem abnormalities: A case report. *J Neuropathol Exp Neurol*. 2005; 64(8):689–694. [PubMed: 16106217]

- Kinney HC, Randall LL, Sleeper LA, Willinger M, Belliveau RA, Zec N, Rava LA, Dominici L, Iyasu S, Randall B, Habbe D, Wilson H, Mandell F, McClain M, Welty TK. Serotonergic brainstem abnormalities in Northern Plains Indians with the sudden infant death syndrome. *J Neuropathol Exp Neurol*. 2003; 62(11):1178–1191. [PubMed: 14656075]
- Kinney HC, Richerson GB, Dymecki SM, Darnall RA, Nattie EE. The brainstem and serotonin in the sudden infant death syndrome. *Annu Rev Pathol*. 2009; 4:517–550. [PubMed: 19400695]
- Kinney HC, Thach BT. MEDICAL PROGRESS The sudden infant death syndrome. *N Engl J Med*. 2009; 361(8):795–805. [PubMed: 19692691]
- Kumar NN, Velic A, Soliz J, Shi Y, Li K, Wang S, Weaver JL, Sen J, Abbott SB, Lazarenko RM, Ludwig MG, Perez-Reyes E, Mohebbi N, Bettoni C, Gassmann M, Suply T, Seuwen K, Guyenet PG, Wagner CA, Bayliss DA. PHYSIOLOGY. Regulation of breathing by CO₂ requires the proton-activated receptor GPR4 in retrotrapezoid nucleus neurons. *Science*. 2015; 348(6240):1255–1260. [PubMed: 26068853]
- Lalley PM, Benacka R, Bischoff AM, Richter DW. Nucleus raphe obscurus evokes 5-HT-1A receptor-mediated modulation of respiratory neurons. *Brain Res*. 1997; 747(1):156–159. [PubMed: 9042541]
- Landis, S. Environmental Influences on the Development of Sympathetic Neurons. In: Bottenstein, J., Sato, G., editors. *Cell Culture in the Neurosciences*. Springer; US: 1985. p. 169-192.
- Li A, Nattie E. Serotonin transporter knockout mice have a reduced ventilatory response to hypercapnia (predominantly in males) but not to hypoxia. *J Physiol*. 2008; 586(9):2321–2329. [PubMed: 18356199]
- Mulkey DK, Rosin DL, West G, Takakura AC, Moreira TS, Bayliss DA, Guyenet PG. Serotonergic neurons activate chemosensitive retrotrapezoid nucleus neurons by a pH-independent mechanism. *J Neurosci*. 2007a; 27(51):14128–14138. [PubMed: 18094252]
- Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA, Guyenet PG. Respiratory control by ventral surface chemoreceptor neurons in rats. *Nature neurosci*. 2004; 7(12):1360–1369. [PubMed: 15558061]
- Mulkey DK, Talley EM, Stornetta RL, Siegel AR, West GH, Chen X, Sen N, Mistry AM, Guyenet PG, Bayliss DA. TASK channels determine pH sensitivity in select respiratory neurons but do not contribute to central respiratory chemosensitivity. *J Neurosci*. 2007b; 27(51):14049–14058. [PubMed: 18094244]
- Nattie EE, Li A, Richerson GB, Lappi DA. Medullary serotonergic neurones and adjacent neurones that express neurokinin-1 receptors are both involved in chemoreception in vivo. *J Physiol*. 2004; 556(Pt 1):235–253. [PubMed: 14724193]
- Niebert M, Vogelgesang S, Koch UR, Bischoff AM, Kron M, Bock N, Manzke T. Expression and function of serotonin 2A and 2B receptors in the mammalian respiratory network. *PLoS One*. 2011; 6(7):e21395. [PubMed: 21789169]
- Ozawa Y, Okado N. Alteration of serotonergic receptors in the brain stems of human patients with respiratory disorders. *Neuropediatrics*. 2002; 33(3):142–149. [PubMed: 12200744]
- Paterson DS, Rivera KD, Broadbelt KG, Trachtenberg FL, Veal RAB, Holm IA, Haas EA, Stanley C, Krous HF, Kinney HC, Markianos K. Lack of association of the serotonin transporter polymorphism with the sudden infant death syndrome in the San Diego dataset. *Pediatric Res*. 2010; 68(5):409–413.
- Paterson DS, Trachtenberg FL, Thompson EG, Belliveau RA, Beggs AH, Darnall R, Chadwick AE, Krous HF, Kinney HC. Multiple serotonergic brainstem abnormalities in sudden infant death syndrome. *JAMA*. 2006; 296(17):2124–2132. [PubMed: 17077377]
- Pena F, Ramirez JM. Endogenous activation of serotonin-2A receptors is required for respiratory rhythm generation in vitro. *J Neurosci*. 2002; 22(24):11055–11064. [PubMed: 12486201]
- Penatti EM, Barina AE, Raju S, Li A, Kinney HC, Commons KG, Nattie EE. Maternal dietary tryptophan deficiency alters cardiorespiratory control in rat pups. *J Appl Physiol*. 2011; 110(2):318–328. [PubMed: 20966190]
- Pineda J, Aghajanian GK. Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. *Neuroscience*. 1997; 77(3):723–743. [PubMed: 9070748]

- Ptak K, Yamanishi T, Aungst J, Milesu LS, Zhang R, Richerson GB, Smith JC. Raphe neurons stimulate respiratory circuit activity by multiple mechanisms via endogenously released serotonin and substance P. *J Neurosci*. 2009; 29(12):3720–3737. [PubMed: 19321769]
- Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, Leiter JC. Neonatal maturation of the hypercapnic ventilatory response and central neural CO₂ chemosensitivity. *Respir Physiol Neurobiol*. 2005; 149(1–3):165–179. [PubMed: 15876557]
- Ramanantsoa N, Hirsch MR, Thoby-Brisson M, Dubreuil V, Bouvier J, Ruffault PL, Matrot B, Fortin G, Brunet JF, Gallego J, Goridis C. Breathing without CO₂ chemosensitivity in conditional Phox2b mutants. *J Neurosci*. 2011; 31(36):12880–12888. [PubMed: 21900566]
- Ramanantsoa N, Vaubourg V, Matrot B, Vardon G, Dauger S, Gallego J. Effects of temperature on ventilatory response to hypercapnia in newborn mice heterozygous for transcription factor Phox2b. *Am J Physiol Regul Integr Comp Physiol*. 2007; 293(5):R2027–2035. [PubMed: 17715184]
- Ramirez JM, Viemari JC. Determinants of inspiratory activity. *Respir Physiol Neurobiol*. 2005; 147(2–3):145–157. [PubMed: 15964786]
- Ray RS, Corcoran AE, Brust RD, Kim JC, Richerson GB, Nattie E, Dymecki SM. Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. *Science*. 2011; 333(6042):637–642. [PubMed: 21798952]
- Richerson GB. Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci*. 2004; 5(6):449–461. [PubMed: 15152195]
- Richerson GB, Messer C. Effect of composition of experimental solutions on neuronal survival during rat brain slicing. *Exp Neurol*. 1995; 131(1):133–143. [PubMed: 7895807]
- Robinson DM, Peebles KC, Kwok H, Adams BM, Clarke LL, Woollard GA, Funk GD. Prenatal nicotine exposure increases apnoea and reduces nicotinic potentiation of hypoglossal inspiratory output in mice. *J Physiol-London*. 2002; 538(3):957–973. [PubMed: 11826179]
- Ruffault PL, D'Autreaux F, Hayes JA, Nomaksteinsky M, Autran S, Fujiyama T, Hoshino M, Hagglund M, Kiehn O, Brunet JF, Fortin G, Goridis C. The retrotrapezoid nucleus neurons expressing Atoh1 and Phox2b are essential for the respiratory response to CO(2). *Elife*. 2015; 4
- Scott MM, Wylie CJ, Lerch JK, Murphy R, Lobur K, Herlitze S, Jiang W, Conlon RA, Strowbridge BW, Deneris ES. A genetic approach to access serotonin neurons for in vivo and in vitro studies. *Proc Natl Acad Sci U S A*. 2005; 102(45):16472–16477. [PubMed: 16251278]
- Serra A, Brozoski D, Hedin N, Franciosi R, Forster HV. Mortality after carotid body denervation in rats. *J Appl Physiol*. 2001; 91(3):1298–1306. [PubMed: 11509529]
- Spyer KM, Thomas T. Sensing arterial CO(2) levels: a role for medullary P2X receptors. *J Auton Nerv Syst*. 2000; 81(1–3):228–235. [PubMed: 10869726]
- Stunden CE, Filosa JA, Garcia AJ, Dean JB, Putnam RW. Development of in vivo ventilatory and single chemosensitive neuron responses to hypercapnia in rats. *Resp physiol*. 2001; 127(2–3):135–155.
- Teppema LJ, Veening JG, Kranenburg A, Dahan A, Berkenbosch A, Olivier C. Expression of c-fos in the rat brainstem after exposure to hypoxia and to normoxic and hyperoxic hypercapnia. *J Comp Neurol*. 1997; 388(2):169–190. [PubMed: 9368836]
- Teran FA, Massey CA, Richerson GB. Serotonin neurons and central respiratory chemoreception: where are we now? *Prog Brain Res*. 2014; 209:207–233. [PubMed: 24746050]
- Veasey SC, Fornal CA, Metzler CW, Jacobs BL. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. *J Neurosci*. 1995; 15(7):5346–5359. [PubMed: 7623157]
- Wang W, Bradley SR, Richerson GB. Quantification of the response of rat medullary raphe neurones to independent changes in pH(o) and P(CO₂). *J Physiol*. 2002; 540(Pt 3):951–970. [PubMed: 11986382]
- Wang W, Pizzonia JH, Richerson GB. Chemosensitivity of rat medullary raphe neurones in primary tissue culture. *J Physiol*. 1998; 511(Pt 2):433–450. [PubMed: 9706021]
- Wang W, Richerson GB. Development of chemosensitivity of rat medullary raphe neurons. *Neuroscience*. 1999; 90(3):1001–1011. [PubMed: 10218799]
- Wang W, Tiwari JK, Bradley SR, Zaykin RV, Richerson GB. Acidosis-stimulated neurons of the medullary raphe are serotonergic. *J Neurophysiol*. 2001; 85(5):2224–2235. [PubMed: 11353037]

- Wickstrom R, Hokfelt T, Lagercrantz H. Development of CO₂-response in the early newborn period in rat. *Respir Physiol Neurobiol.* 2002; 132(2):145–158. [PubMed: 12161328]
- Zhao Z-Q, Scott M, Chiechio S, Wang J-S, Renner KJ, Gereau RW, Johnson RL, Deneris ES, Chen Z-F. Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J Neurosci.* 2006; 26(49):12781–12788. [PubMed: 17151281]

Highlights

Early postnatal ages 5-HT neurons provide tonic drive to the respiratory network only

Early postnatal ages 5-HT neurons does not contribute to chemoreception.

5-HT neurons from P21 animals switch their role from tonic drive to chemoreceptors.

Our results could be related to the ventilatory abnormalities proposed in SIDS.

Figure 1.

During early development, normal eupneic breathing in room air was impaired in *Lmx1b*^{f/f/p} mice, and there was a minimal response to hypercapnia in both WT and *Lmx1b*^{f/f/p} mice. Examples of plethysmography recordings from a single WT (**traces on left**) and a single *Lmx1b*^{f/f/p} mouse (**traces on right**) at the age of P4 in room air (**A**), 7% CO₂ (**B**) and 10% O₂ (**C**). Scale bar is 2 sec.

Figure 2.

Breathing in room air was impaired in *Lmx1b^{f/f/p}* compared to WT mice early in development, but normalized after 2 weeks of age. Plethysmography recordings from P1–P21 mice in room air were used to measure minute ventilation (A), minute ventilation to oxygen consumption ratio (B), tidal volume (C) and respiratory frequency (D). *, **, *** and **** comparing genotypes; p < 0.05, 0.01, 0.001 and 0.0001 respectively.

Figure 3.

Age dependence of the response to hypercapnia and hypoxia of *Lmx1b^{f/fp}* and WT mice. The response to 7% CO₂ was measured from P1–P21 as the change in minute ventilation (A), and the change in minute ventilation to oxygen consumption ratio (B). *Lmx1b^{f/fp}* mice lacked the normal increase in HCVR that occurred in WT mice after P12. The response to 10% O₂ was also measured from P1–P21 as the change in minute ventilation (C), and minute ventilation to oxygen consumption ratio (D). *Lmx1b^{f/fp}* mice had a smaller increase in the ventilatory response to 10% O₂ at P12 and P21 compared to WT mice, but there was no difference in V_E/VO_2 at any age. * and *** comparing genotypes; $p < 0.05$ and 0.001 respectively.

Figure 4.

Chemosensitivity of 5-HT neurons increased with age in culture. Patch-clamp recordings revealed that the percentage of 5-HT neurons stimulated by acidosis (**A**), the chemosensitivity index (**B**) and the percentage change in firing rate induced by acidosis (**C**) increased with age over the first 3–4 weeks, whereas the percentage of 5-HT neurons inhibited by acidosis decreased (**A**). For **B** & **C** the mean values were calculated from all neurons, including those that were not chemosensitive. The number of neurons for each group are as listed in Table 2. *, *** and ****; $p < 0.05$, 0.001 and 0.0001 respectively.

Figure 5.

Electrophysiological properties of 5-HT neurons *in vitro*. Shown are changes of membrane capacitance (**A1**), afterhyperpolarization amplitude (AHP) (**A2**), resting membrane potential (**B1**) and action potential amplitude (**B2**) at different ages during development in culture. For B1 and B2, values of n are the same as those in Fig. 4A. *, ** and ****; $p < 0.05$, 0.01 and 0.0001 respectively.

Figure 6.

Growth rate of *Lmx1b*^{f/fp} mice was decreased compared to WT mice, and was worsened by hypoxia in both genotypes (A). Concomitant hypercapnia and hypoxia improved the growth rate of WT mice (B) but had no effect on *Lmx1b*^{f/fp} mice (not shown). Shown are growth curves for WT (solid lines) and *Lmx1b*^{f/fp} (dashed lines) mice in normoxia and hypoxia (A), and in WT mice in hypoxia plus hypercapnia (B). Symbols above the data points represent statistical comparison of groups as shown in the keys in the upper right corner of each graph. Symbols are plotted above each curve at the first data point at which statistical significance is reached at p values of <0.05 (open symbol), <0.01 (light gray symbol), <0.001 (dark gray symbol) and <0.0001 (black symbol), respectively.

Figure 7.

Mortality was increased by hypoxia (A), decreased by hyperoxia (A) and partially rescued by concomitant hypercapnia (B). Survival curves from *Lmx1b*^{f/f/p} mice (dashed lines) and their WT littermates (solid lines). Values of n at the start of the experiment were 53 (WT normoxia), 25 (*Lmx1b*^{f/f/p} normoxia), 58 (WT hypoxia), 58 (*Lmx1b*^{f/f/p} hypoxia), 35 (WT hyperoxia), 49 (*Lmx1b*^{f/f/p} hyperoxia), 36 (WT hypercapnic hypoxia) & 16 (*Lmx1b*^{f/f/p} hypercapnic hypoxia). Detailed statistics are presented in table 3.

Table 1

Development of ventilatory response to 7% CO₂ and 10% O₂ from WT and Lmx1b^{fl/p} mice at different postnatal ages.

Response to 7% CO2				Response to 10% O2				
Age	V _T (%change)		f _R (%change)		V _T (%change)		f _R (%change)	
	WT	Lmx1b ^{fl/p}	WT	Lmx1b ^{fl/p}	WT	Lmx1b ^{fl/p}	WT	Lmx1b ^{fl/p}
P1	145±7	173±7	118±7	129±3 *	105±4	125±3 *	120±6	129±5
P4	172±11	168±10	142±10	205±32 ***	131±10	108±6	144±8	139±16
P7	174±11	176±9	115±3	177±14	151±8	144±12	130±6	161±19 *
P12	201±7	152±8 **	144±4	157±6	103±2	91±3	147±6	120±3
P21	237±14	164±10 ***	197±7	189±9	118±7	98±4	152±8	150±7

Values are means ± SEM. V_T: tidal volume, f_R: respiratory frequency,

*, **, ***, *****, P <0.05, <0.01, <0.001, <0.0001, respectively.

Two-way ANOVA, Bonferroni post-Hoc test, comparing WT to Lmx1b^{fl/p} at each age.

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Table 2

Changes in chemosensitivity of 5-HT neurons in primary dissociated cell culture.

Age Range	N	Mean Age	CI	% Change in FR	% of Neurons That Are Stimulated by Acidosis	% of Neurons That Are Inhibited by Acidosis
1-6	19	3.4	84	-19	11	68
7-9	14	7.7	127	25		
10-12	17	10.9	130	37	35	26
13-16	11	13.6	158	79		
17-21	19	18.7	159	77	53	27
22-30	34	24.9	282	199		
31-40	42	36.5	306	211	75	13
41-50	43	46.0	268	262		
51-70	29	59.3	263	283	79	3

Table 3

Survival curves. Cox proportional hazards test results.

Effect of genotype condition.		
Groups	HR (95% CI)	P
Normoxia; Lmx1b ^{fl/p} vs WT	1.54 (0.502–4.472)	0.449
Hypoxia; Lmx1b ^{fl/p} vs WT	1.61 (1.007–2.561)	0.046
Hyperoxia ; Lmx1b ^{fl/p} vs WT	0.38 (0.107–1.345)	0.194
Hypercapnia-hypoxia; Lmx1b ^{fl/p} vs WT	0.55 (0.242–1.275)	0.165
Effect of environmental condition.		
Groups	HR (95% CI)	P
WT; Hypoxia vs Normoxia	3.77 (2.023–7.008)	0.0001
Lmx1b ^{fl/p} ; Hypoxia vs Normoxia	3.36 (1.831–6.171)	0.0001
WT; Hypoxia vs Hypoxia-Hypercapnia	2.27 (1.274–4.069)	0.006
Lmx1b ^{fl/p} ; Hypoxia vs Hypoxia-Hypercapnia	2.07 (1.165–3.677)	0.01
WT; Hypoxia-Hypercapnia vs Normoxia	1.844 (0.816–4.166)	0.141
Lmx1b ^{fl/p} ; Hypoxia-Hypercapnia vs Normoxia	1.985 (0.756–5.201)	0.164
WT; Hyperoxia vs Normoxia	3.18 (0.96–10.56)	0.112
Lmx1b; Hyperoxia vs Normoxia	1.76 (0.56–5.48)	0.28

HR indicates hazard ratio; CI, confidence interval.